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Methanol Oxidation Genes in the Marine Methanotroph *Methylomonas* sp. Strain A4

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Methanol dehydrogenase has been purified from the type I marine methanotroph *Methylomonas* sp. strain A4 and found to be similar to other methanol dehydrogenase enzymes in subunit composition, molecular mass, and N-terminal sequence of the two subunits. A heterologous gene probe and a homologous oligonucleotide have been used to identify a DNA fragment from *Methylomonas* sp. strain A4 which contains *moxF*, the gene encoding the large subunit of methanol dehydrogenase. Protein expression experiments with *Escherichia coli*, immunoblotting of expression extracts, and partial DNA sequence determination have confirmed the presence of *moxF* on this DNA fragment. In addition, expression and immunoblot experiments have shown the presence of the genes for the small subunit of methanol dehydrogenase (*moxI*) and for the methanol dehydrogenase-specific cytochrome *c* (*moxG*). The *moxG* gene product has been shown to be cytochrome c_{552}. The expression experiments have also shown that two other genes are present on this DNA fragment, and our evidence suggests that these are the homologs of *moxJ* and *moxR*, whose functions are unknown. Our data suggest that the order of these genes in *Methylomonas* sp. strain A4 is *moxFJGIR*, the same as in the facultative methylotrophs. The transcriptional start site for *moxF* was mapped. The sequence 5′ to the transcriptional start does not resemble other promoter sequences, including the putative *moxF* promoter sequence of facultative methylotrophs. These results suggest that although the order of these genes and the N-terminal amino acid sequence of MoxF and MoxI are conserved between distantly related methylotrophs, the promoters for this gene cluster differ substantially.

Gram-negative methylotrophic bacteria oxidize methanol via a periplasmic methanol dehydrogenase, which contains the quinone cofactor pyrrolo-quinoline quinone and utilizes a soluble cytochrome *c* (called cytochrome c_L) as an electron acceptor (5). This methanol oxidation system is complex, and more than 20 genes (*mox* genes) that are required for methanol dehydrogenase regulation, synthesis, and assembly and for synthesis of the cofactor pyrrolo-quinoline quinone (19) have been identified in the facultative methanol utilizer *Methylobacterium extorquens* AM1.

The structural genes for the methanol dehydrogenase alpha and beta subunits (*moxF* and *moxI*, respectively) have been cloned from the facultative methanol utilizers *M. extorquens* AM1 (28, 29), *Methylobacterium organophilum* XX (24), and *Paracoccus denitrificans* (16). In all three cases, the genes have been found to reside in a gene cluster together with the gene for cytochrome c_L (*moxG*) and another gene of unknown function (*moxJ*) (19). The order of these genes in all three bacteria is *moxFJGI*. Transcriptional start sites for this gene cluster have been mapped for the two *Methylobacterium* strains (4, 23), and a comparison of upstream sequences has identified a putative promoter sequence for these genes as follows: AAAGACA–18 bp–TAGAAA–4 to 5 bp–+1 (19).

Although most of the genetic studies of the *mox* system have focused on facultative methanol-utilizing bacteria, some information is available for *mox* genes in methane-utilizing bacteria (methanotrophs). That work has centered on *moxF*, since this gene is apparently conserved among a wide range of methylotrophic bacteria. The *moxF* genes have been sequenced from the three facultative methanol utilizers noted above and show strong similarity at both amino acid and nucleotide levels (4, 16, 23). In addition, DNA probing studies have shown that *moxF* genes from *Methylobacterium* strains hybridize to DNA from a variety of methanol- and methane-utilizing bacteria (6, 33). DNA containing *moxF* has been cloned from four methanotrophs by using heterologous probes (33), expression cloning (1), or mutant complementation (6), but no further studies have been carried out. Mutant complementation suggests that in the type II methanotroph *Methylosporovibrio methanica*, genes equivalent to *moxF*, *moxG*, and *moxI* are clustered on a DNA fragment, but it is not known whether the *moxJ* equivalent is also present (6).

We are interested in studying *mox* genes in methanotrophic bacteria in order to analyze regulatory mechanisms for key methylotrophic functions. Methanotrophs are an important group of bacteria from both commercial and environmental points of view (5, 19). Information on the molecular biology of key methylotrophic functions in these bacteria is essential for understanding and manipulating their unique metabolic activities. In this article, we report the cloning and analysis of the *moxF* region in a type I marine methanotroph, *Methylomonas* sp. strain A4. This strain falls into the γ subgroup of the *Proteobacteria* (8) and as such is more distant evolutionarily than the other strains for which *mox* genes have been studied, which all are found in the α subgroup (8). Although the gene order was found to be

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant trait(s)	Reference or source
Methylomonas sp. strain A4	Type I marine methanotroph	20
E. coli		
DH5α	$r^- m^-$ *recA1*	Bethesda Research Laboratories, Inc.
HB101	*recA strR*	7
CSR603	*recA uvrA6*	31
Plasmids		
pRK2013	Km^r (mobilizing plasmid)	14
pDA4628	Tc^r Km^r IncP1 cosmid	2
pT7-3	Ap^r T7 promoter	S. Tabor
pT7-4	Ap^r T7 promoter	S. Tabor
pT7-5	Ap^r T7 promoter	35
pT7-6	Ap^r T7 promoter	35
pGP1-2	Km^r *c*I857; T7 RNA polymerase gene	35
pADS234	*Methylomonas* sp. strain A4 10.5-kb *Hin*dIII (*moxF*) fragment in pDA4628	This study
pAD234M4	*Methylomonas* sp. strain A4 5.7-kb *Hin*dIII-*Eco*RI (*moxF*) fragment in pDA4628	This study
pDW8B	10.5-kb *Hin*dIII (*moxF*) fragment in pT7-6 (orientation B[a])	This study
pDW8A	10.5-kb *Hin*dIII (*moxF*) fragment in pT7-6 (orientation A[b])	This study
pDW11	2.2-kb *Eco*RI *moxF'* in pT7-6 (orientation B)	This study
pDW12	2.2-kb *Eco*RI *moxF'* in pT7-6 (orientation A)	This study
pDW33	3.5-kb *Eco*RI *moxF'* in pT7-6 (orientation B)	This study
pDW13	3.5-kb *Eco*RI *moxF'* in pT7-6 (orientation A)	This study
pDW33ΔBal	Deletion of proximal *Bal*I fragment from pDW33	This study
pDW33ΔSph	Deletion of proximal *Sph*I fragment from pDW33	This study

[a] Orientation B, left to right as shown in Fig. 1 with respect to the T7 promoter of the vector.
[b] Orientation A, right to left as shown in Fig. 1 with respect to the T7 promoter of the vector.

similar to that in the other methylotrophs, the transcription start site suggests that the promoter in this system is quite different from that in the *Methylobacterium* strains.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Methylomonas* sp. strain A4 (20) was grown in NMS mineral salts solution (39) plus 1.5% (wt/vol) NaCl at 37°C under an atmosphere of 20% methane and 80% air (vol/vol). *Escherichia coli* strains were grown at 37 or 30°C on Luria broth (25). Where appropriate, filter-sterilized antibiotic solutions were added to sterile medium in the following concentrations: ampicillin, 50 μg/ml; kanamycin, 50 μg/ml; rifamycin, 10 μg/ml; tetracycline, 10 μg/ml. When kanamycin and ampicillin were used together, the concentrations were 40 μg/ml each.

Purification of methanol dehydrogenase from *Methylomonas* sp. strain A4. All isolation steps were performed at 0 to 4°C. Freeze-thawed *Methylomonas* sp. strain A4 cells were resuspended in buffer A (10 mM Tris-HCl, pH 8.0) and passed through a French pressure cell three times at 20,000 lb/in^2. The cell slurry was centrifuged at 13,200 × *g* for 30 min to remove unlysed cells and cell debris. The supernatant was centrifuged at 15,000 × *g* for 90 min. The supernatant was brought to 30% of saturation in solid ammonium sulfate, stirred for 3 h, and centrifuged at 13,200 × *g* for 15 min. The pellet was discarded, and the concentration of ammonium sulfate in the supernatant was raised to 60% saturation. The solution was then stirred for 3 h and centrifuged for 15 min at 13,200 × *g*, and the pellet was resuspended in a minimal volume of buffer A (30 to 60% ammonium sulfate fraction). The proteins precipitating in 30 to 60% ammonium sulfate were dialyzed against three changes of buffer A and loaded on a DEAE-cellulose column (2.5 by 36 cm) equilibrated with buffer A. The column was washed with approximately two bed volumes each of buffer A, buffer B (50 mM Tris-HCl, pH 8.0), and buffer C (50 mM Tris-HCl, 50 mM KCl, pH 8.0); the methanol dehydrogenase was then eluted with buffer D (50 mM Tris-HCl, 100 mM KCl, pH 8.0). This sample was dialyzed against three changes of buffer A and loaded on a DEAE-Sepharose CL-6B column (2.5 by 30 cm) equilibrated with buffer A. After washes with two bed volumes each of buffers A, C, and D, the sample was eluted with buffer E (50 mM Tris-HCl, 200 mM KCl, pH 8.0). The methanol dehydrogenase sample was concentrated with a stirred cell (YM30 filter) and loaded on a Sephadex G-200 column (2.5 by 40 cm) equilibrated with buffer E. The eluting methanol dehydrogenase was again concentrated with a stirred cell and resolved on a preparative isoelectric focusing bed (10 by 20 cm; LKB, Bromma, Sweden) containing 4 Ultrodex; 1.0% ampholyte, pH 4.0 to 6.0; and 1% ampholyte, pH 5.0 to 8.0. Methanol dehydrogenase migrated as a single band focusing at pH 5.5. This band was eluted from the Ultrodex with buffer B, dialyzed against three changes of buffer B, and concentrated with an Amicon Centricon (Diaflow Corp., Amicon) YM30 filter. Protein assay was as described in reference 22.

Amino acid sequence determination. The N-terminal amino acid sequences of the methanol dehydrogenase alpha and beta subunits were determined with a gas-phase peptide sequencer (Applied Biosystems 477A).

Antiserum production and isolation of immunoglobulin G fraction. Antiserum to the methanol dehydrogenase purified from *Methylomonas* sp. strain A4 was generated by Cocalico Biologicals, Inc., Reamstown, Pa., as described previously (33). The antiserum from each rabbit was pooled and purified as described by Nowotny (27).

Protein electrophoresis and immunoblotting. Sodium dodecyl sulfate (SDS) and native polyacrylamide gel electrophoreses were performed by the procedure of Laemmli (18) on 12 to 18% (wt/vol) acrylamide gels. Proteins were blotted onto nitrocellulose membranes with the Trans-Blot system (Bio-Rad Laboratories, Richmond, Calif.) per the manufacturer's directions. After treatment with serum raised against purified protein, filter-bound antibodies were detected with the alkaline phosphatase assay from Bio-Rad. Prestained protein molecular mass standards were obtained from Sigma Chemical Co., St. Louis, Mo., and were as follows (in daltons): α-macroglobulin, 180,000; β-galactosidase, 116,000; fructose-6-phosphate kinase, 84,000; pyruvate kinase, 58,000; fumarase, 48,500; lactic dehydrogenase, 36,500; triosephosphate isomerase, 26,600.

DNA manipulations. Total cellular DNA was isolated from *Methylomonas* sp. strain A4 as described previously (15). Large- and small-scale plasmid preparations were isolated by standard methods (25), which included two rounds of CsCl-ethidium bromide density gradient purification of the bulk preparations.

Restriction endonucleases and other DNA enzymes (ligase, kinase, etc.) were obtained from Bethesda Research Laboratories (Rockville, Md.) or New England Biolabs, Inc. (Beverly, Mass.) and used according to the manufacturer's suggestions.

Agarose gel electrophoresis, ligations, and transformations were performed as described by Maniatis et al. (25).

Radiolabelling of probe DNA was performed by the procedure of Rigby et al. (30).

DNA-DNA hybridizations. DNA-DNA hybridizations were performed directly on dried agarose gels (26) with modifications described previously (33).

Colony hybridizations were performed as described by Maniatis et al. (25).

Cosmid clone bank constructions. Genomic DNA from *Methylomonas* sp. strain A4 was partially digested with *Hin*dIII and combined with a complete *Hin*dIII digest of genomic DNA. This combination ensures a more complete representation of the larger *Hin*dIII fragments in the bank. The DNA was fractionated to sizes between 10 and 20 kb on sucrose gradients (25). Cosmid vector pDA4628 (2) was digested with *Hin*dIII and ligated with the fractionated *Methylomonas* sp. strain A4 DNA in a 1:4 ratio (vector to insert); the total DNA concentration was 900 μg/ml. The ligated DNA was packaged in vitro with lambda procapsids (34). This reaction mixture was used to infect *E. coli* HB101, selecting for tetracycline resistance. A pool of approximately 17,000 transductants was obtained, with an average insert size of 13 kb.

Nucleotide sequencing. Dideoxy DNA sequencing reactions (32) were performed with the Sequenase and Sequenase II systems from U.S. Biochemical Corp. (Cleveland, Ohio). Templates for the reactions were double-stranded plasmids prepared as described by Chen and Seeberg (9). Primers were either purchased (Universal and T7 primers) from U.S. Biochemical Corp. or synthesized by the Caltech Microchemical Facility. The internal primer A4M1 was 5′-GTYTGCATNACCCARAC-3′ (Y = T/C, N = T/C/A/G, R = A/G).

Protein expression. Protein expression from cloned genes in *E. coli* DH5α was analyzed with T7 promoter vectors pT7-3, pT7-4, pT7-5, and pT7-6 and plasmid pGP1-2 carrying the T7 RNA polymerase gene as described by Tabor (35) and Tabor and Richardson (36). The protocol for [^{35}S]methionine labelling (20 to 30 min of induction) was used for labelled polypeptides, and the protocol for maximum expression (2.5 h of induction in the absence of [^{35}S]methionine) was used for immunoblotting. Protein molecular mass standards were from Bio-Rad and were as follows (in daltons): phosphorylase *b*, 97,400; bovine serum albumin, 66,200; ovalbumin, 42,699; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; lysozyme, 14,400.

RNA isolation. Total RNA was isolated from 15 ml of a culture growing exponentially on methane. Hot lysis buffer (7.5 ml) (20 mM Tris-HCl, pH 7.5; 2% SDS [wt/vol]; 20 mM EDTA; 200 mM NaCl) was added, and the mix was incubated in a boiling water bath for 5 min. Extraction with acid (pH 4.0) phenol was performed three times at 69°C and was followed by one extraction with neutral phenol-chloroform-isoamyl alcohol (25:24:1) and one extraction with chloroform-isoamyl alcohol (24:1). The aqueous phase was diluted by adding 3 volumes of water, and RNA was precipitated by adding 2.5 volumes of ethanol. The pellets were dissolved in water, extracted with ethyl ether, and reprecipitated. Aliquots of RNA solution in water were kept at −70°C.

Primer extension analysis. Two oligonucleotides, 5′-TC CAGGTTATTGTTATGCACTGATTAG-3′ (a) and 5′-CG TAGTCGTCCCACATGACGAGCAAATTTCG-3′ (b), complementary to nucleotides −30 to −7 and 11 to 41, respectively, with respect to the translational start site for *moxF*, were synthesized by the Caltech Microchemical Facility. Superscript reverse transcriptase (GIBCO-BRL) was used for primer extension, and reactions were performed in accordance with the recommendations of the manufacturer, except that nonlabelled dCTP was eliminated from the reaction to increase specific activity. The products of transcription were then precipitated with ethanol, washed with 70% (vol/vol) ethanol, redissolved in formamide loading buffer (80% [vol/vol] formamide, 10 mM EDTA, pH 8.0), heated for 2 min at 80°C, and subjected to electrophoresis on 6% (wt/vol) polyacrylamide gels simultaneously with a sequence ladder generated with the same primer.

Nucleotide sequence accession number. The nucleotide sequence of the DNA surrounding *moxF* reported here has been assigned GenBank accession number L11308.

RESULTS

Purification of methanol dehydrogenase. The methanol dehydrogenase was purified to homogeneity as judged by SDS-polyacrylamide gels. The molecular weight of the enzyme as isolated was 134,000 ± 6,000 as determined on a Sephadex G-200 column (2.5 by 35 cm). The purified methanol dehydrogenase showed two bands on SDS-polyacrylamide gels when the samples were boiled for 2 min in sample buffer containing 1.0% SDS and 0.04% β-mercaptoethanol prior to being loaded on the gel. The two subunits had molecular masses of approximately 59 and 12 kDa and showed an approximate 1:1 molar ratio when stained with Coomassie brilliant blue R and scanned with an LKB 2202 Ultroscan densitometer. Thus, the enzyme had an $\alpha_2\beta_2$ structure, in accordance with other methanol dehydrogenase enzymes (5). The association between the two subunits was strong, since the methanol dehydrogenase ran as one band on native gels. In addition, only a fraction of the sample separated when the methanol dehydrogenase was incubated in sample buffers containing 1.0% SDS and 0.04% β-mercaptoethanol and sonicated in a batch-type sonicator for 10 min at room temperature.

The N-terminal amino acid sequences were determined for both subunits and were as follows: large subunit, NKELDQ

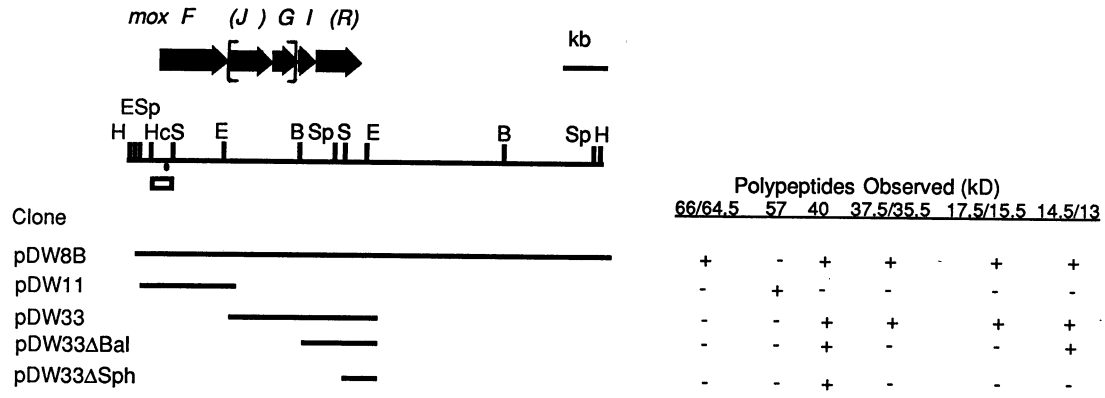

FIG. 1. Map of the 10.5-kb *Hin*dIII fragment in pADS234 that contains the *moxF* gene cluster and results of protein expression experiments. Dot denotes the location of the A4M1 oligonucleotide. Box denotes the region sequenced. The inserts shown are in the clones indicated, in the orientation left to right as shown with respect to the T7 promoter in pT7-6. *moxF* and *moxI* encode the large and small subunits of methanol dehydrogenase, respectively. *moxG* encodes cytochrome c_{552}. The identities of the second and fifth genes cannot be confirmed at this time, but they are proposed to be *moxJ* and *moxR* on the basis of their locations and the sizes of the encoded polypeptides (3, 4, 37). The order of *moxJ* and *moxG* is tentative and is assumed to be the same as in facultative methylotrophs (19). Polypeptide sizes (in kilodaltons for unprocessed and mature forms) for the genes shown are: *moxF*, 66 and 64.5; *moxJ*, 37.5 and 35.5; *moxG*, 17.5 and 15.5; *moxI*, 14.5 and 12.5; *moxR*, 40. Abbreviations: H, *Hin*dIII; E, *Eco*RI; Sp, *Sph*I; Hc, *Hin*cII; S, *Sal*I; B, *Bal*I.

MSRQNTNWVMQT; small subunit, YDGTNCKPGVCWE PKPGY.

Cloning of *moxF*. *Methylomonas* sp. strain A4 genomic 0DNA was digested with *Hin*dIII, separated on agarose gels, and hybridized to ORF9, a DNA fragment of approximately 870 bp containing an internal portion of *moxF* from *M. extorquens* AM1 (28). *moxF* encodes the methanol dehydrogenase large (alpha) subunit. A *Hin*dIII fragment of approximately 11 kb was identified with the ORF9 probe. A cosmid clone bank was constructed in vector pDA4628 with a *Methylomonas* sp. strain A4 genomic DNA sample which contained a mixture of DNA that had been partially digested

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          10         20         30         40         50         60
CGCGCCGAAAATCCAAGGCATTACGTTTTGATCAAGAAAGCTGATTGCTGCAAGTTGACA

          70         80         90        100        110        120
                     *
CAAATAAACACATCGGCGCGTTAAGCTAGGCCTTACTAATCAGTGCATAACAATAACCTG

         130        140        150        160        170        180
GAGGAAGTATGCGGATTTCGAAATTTGCTCGTCATGTGGGGACGACTACGGCGCTGGCGT
      M  R  I  S  K  F  A  R  H  V  G  T  T  T  A  L  A

         190        200        210        220        230        240
CGGGTATCCTGCTGGCCTTGTCGCAACCGGCGCAAGCCAATAAAGAGCTGGACCAGATGT
S  G  I  L  L  A  L  S  Q  P  A  Q  A**N  K  E  L  D  Q  M

         250        260        270        280        290        300
CGCGCCAGAACACCAATTGGGTGATGCAGACCAAGGATTACAGCTCCACGCATTACAGCG
S  R  Q  N  T  N  W  V  M  Q  T  K  D  Y  S  S  T  H  Y  S

         310        320        330        340        350        360
AGTTGTATGACATCAATATCACCAACGTCAAGAACCTGAAGCCAGCCTGGTCTTTTTCAA
E  L  Y  D  I  N  I  T  N  V  K  N  L  K  P  A  W  S  F  S

         370        380        390        400
CCGGCGTGCTGAACGGCCACGAAGGCGGGCCATTGGTCGTCGAC
T  G  V  L  N  G  H  E  G  G  P  L  V  V  D
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FIG. 2. DNA sequence and translated amino acid sequence of the *Methylomonas* sp. strain A4 region surrounding the 5′ portion of *moxF*. The sequence similar to the putative promoter for *moxF* from facultative methylotrophs is underlined, the σ^{70} −35 sequence is double underlined, the transcriptional start site is indicated with an asterisk and boldface type, and the putative ribosome binding site is indicated in boldface type and is double underlined. The amino acids of the signal peptide are in italics, the cleavage site is marked with a double asterisk, and the amino acids that are identical to those determined by protein sequencing are underlined.

and completely digested with *Hin*dIII. Colony blots were screened with the ORF9 probe, and a number of positive clones were all found to contain identical 10.5-kb *Hin*dIII fragments. One of these, designated pADS234, was chosen for further study, and the insert was mapped with a number of restriction enzymes (Fig. 1).

An oligonucleotide of 17 residues with a 16-fold redundancy (A4M1; see Materials and Methods) was synthesized on the basis of part of the N-terminal amino acid sequence (NWVMQT) of the methanol dehydrogenase large subunit from *Methylomonas* sp. strain A4. The oligonucleotide used was based on the noncoding strand, so it could also serve as a sequencing primer for the 5′ end of the gene. This oligonucleotide was found to hybridize to a 0.35-kb *Hin*cII-*Sal*I fragment within the 10.5-kb *Hin*dIII fragment in pADS234 (Fig. 1).

Sequencing of the 5′ region of *moxF*. A 404-bp region of the 10.5-kb *Hin*dIII fragment containing this 0.35-kb *Hin*cII-*Sal*I fragment was sequenced and was found to contain a partial open reading frame of 92 amino acids (Fig. 2). This open reading frame included 18 amino acids that were identical to the N-terminal sequence determined from the methanol dehydrogenase large subunit, confirming that this was the 5′ end of *moxF*. A putative ribosome binding site was identified upstream of the translational start site, and the sequence predicts a leader peptide of 30 amino acids, with a typical leader peptide structure (38). Since the mature form of the *moxF* gene product (the methanol dehydrogenase large subunit) is known to be 59 kDa, *moxF* should extend approximately 0.25 kb past the first *Sal*I site shown in Fig. 1.

Transcriptional start site mapping. Primer extension analysis was used to determine the 5′ end of the *moxF* mRNA. Two oligonucleotides, specific to the beginning of *moxF* and a region directly upstream of *moxF*, respectively, were employed (see Materials and Methods). Both primers produced a major extension product, although a few minor extension products were also observed (Fig. 3). In both cases, the 5′ end of the major *moxF*-specific mRNA mapped to the cytosine 42 bases upstream of the translational start site for *moxF* (Fig. 2 and 3). No known consensus promoter sequences (11, 17) were present immediately 5′ to this start site.

Protein expression. Polypeptides encoded by the 10.5-kb *Hin*dIII fragment containing *moxF* were analyzed by protein expression in *E. coli* with a T7 expression system. The clones analyzed are shown in Fig. 1. In one orientation (left to right as shown in Fig. 1), nine major polypeptides were expressed from the 10.5-kb *Hin*dIII fragment that were not present with controls containing vector alone or with the *Hin*dIII fragment in the opposite orientation with respect to the T7 promoter (Fig. 1). Eight of these polypeptides were present in pairs with a difference in mass of 1.5 to 2 kDa, as expected for unprocessed and mature forms of periplasmic polypeptides (10). These included a pair at 66 and 64.5 kDa, another at 37.5 and 35.5 kDa, a third at 17.5 and 15.5 kDa, and a fourth at 14.5 and 13 kDa. The ninth polypeptide was 40 kDa.

The 66- and 64.5-kDa polypeptides are approximately the correct size to be the methanol dehydrogenase large subunit and were expressed only from the clone that should contain the intact *moxF*. Approximately 1.6 kb of DNA should be required to encode *moxF*; therefore, the second *Eco*RI fragment shown in Fig. 1 should cut off a 3′ fragment of approximately 0.3 kDa. A clone containing this *Eco*RI fragment (pDW11) expressed a polypeptide of 57 kDa, which

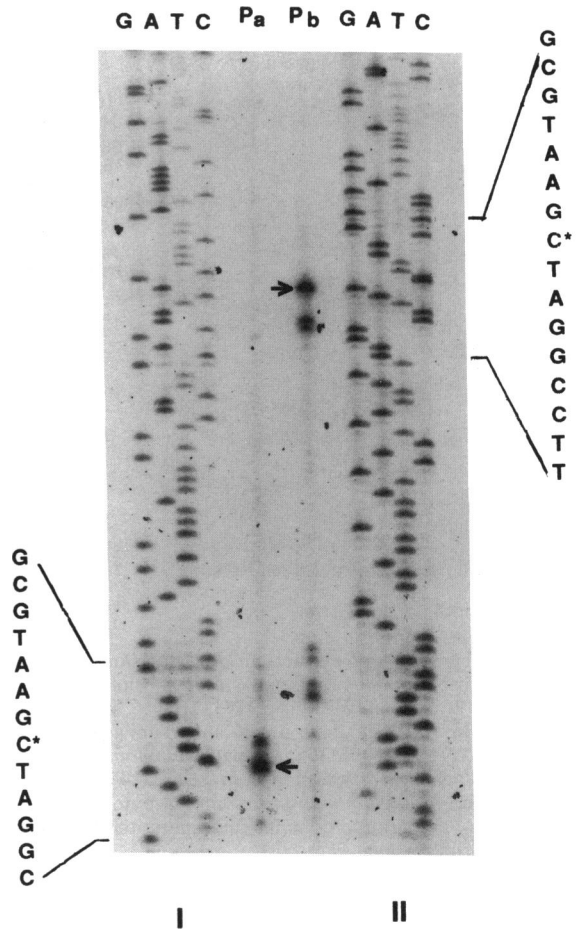

FIG. 3. Primer extension analysis of *moxF*-specific mRNA. Primers a (panel I) and b (panel II) were annealed with total RNA from *Methylomonas* sp. strain A4 grown on methane, extended with reverse transcriptase, and coelectrophoresed with DNA sequencing reactions (lanes G, A, T, and C) initiated with the same oligonucleotide. Lanes P_a and P_b, primer extension reactions with primers a and b, respectively. Major products of the primer extension reaction are indicated by arrows. The DNA sequences of the sense strand are shown to indicate the transcription start point, denoted with an asterisk.

is the approximate size predicted for a truncated form of the 66-kDa polypeptide.

The polypeptides at 37.5 and 35.5 kDa and at 17.5 and 15.5 kDa were all expressed from two clones only: pDW8B, containing the 10.5-kb *Hin*dIII fragment, and pDW33, containing an internal 3.5-kb *Eco*RI fragment. The first two polypeptides are the correct sizes to be the *moxJ* gene product, the mature form of which is approximately 30 kDa in *M. extorquens* AM1 and *P. denitrificans* (3, 37). The second two polypeptides are approximately the correct sizes to be the *moxG* gene product, the methanol dehydrogenase-specific cytochrome *c*. In *Methylomonas* sp. strain A4, MoxG has been proposed to be the 14-kDa cytochrome c_{552} (13). Although our data do not allow ordering of the *Methylomonas* sp. strain A4 genes encoding these two polypeptides, in *M. extorquens* AM1 and *P. denitrificans* the order is *moxJ-moxG* (3, 37).

The polypeptides at 14.5 and 13 kDa were expressed from three clones: pDW8B, pDW33, and, in addition, pDW33Δ

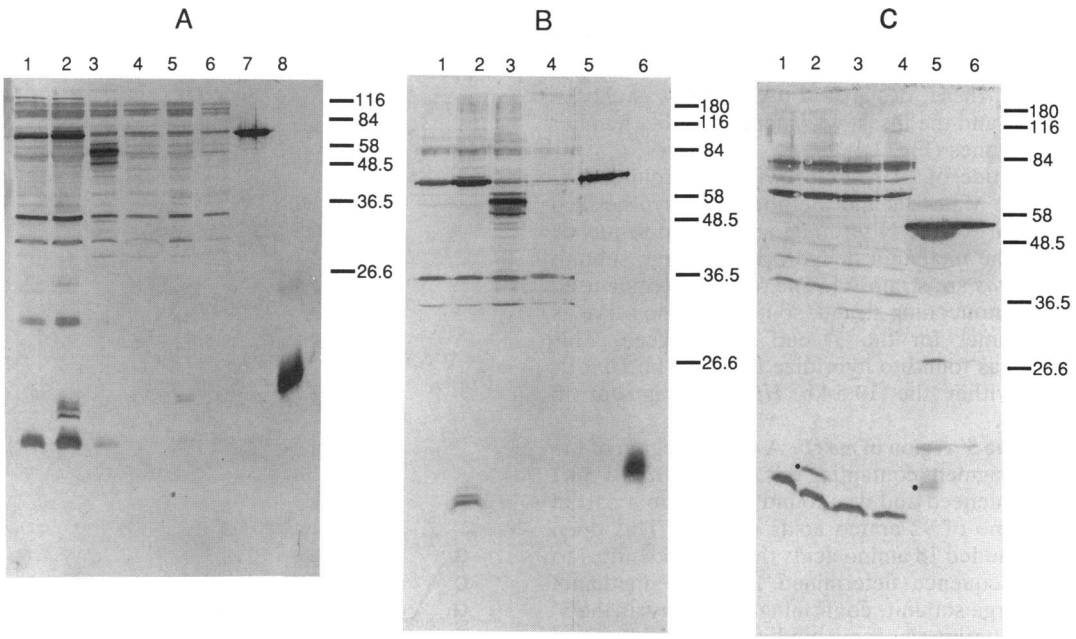

FIG. 4. Immunoblots of extracts from expression experiments with *E. coli* strains containing the plasmids noted. (A) Incubation with antiserum against *Methylomonas* sp. strain A4 holomethanol dehydrogenase (containing both MoxF and MoxI). Lanes: 1, pDW8A; 2, pDW8B; 3, pDW11; 4, pDW33; 5, pDW33ΔBal; 6, pDW33ΔSph; 7, purified *Methylomonas* sp. strain A4 MoxF; 8, purified *Methylomonas* sp. strain A4 MoxI. (B) Incubation with antiserum against *Methylomonas* sp. strain A4 holomethanol dehydrogenase. Lanes: 1, pDW8A; 2, pDW8B; 3, pDW11; 4, pDW33; 5, purified *Methylomonas* sp. strain A4 MoxF; 6, purified *Methylomonas* sp. strain A4 MoxI. (C) Incubation with antiserum against *Methylomonas* sp. strain A4 cytochrome c_{552}. Lanes: 1, pDW8A; 2, pDW8B; 3, pDW11; 4, pDW33; 5, pDW33ΔBal; 6 and 7, purified *Methylomonas* sp. strain A4 cytochrome c_{552}. Standards (in kilodaltons) are indicated on the right of each panel. The cytochrome c_{552} preparation contains multimers; the monomer is marked with dots.

Bal, containing the 1.6-kb *Bal*I-*Eco*RI fragment shown in Fig. 1. These polypeptides are approximately the correct size to be the *moxI* gene product, the methanol dehydrogenase small subunit, which is present immediately downstream of *moxG* in a number of methylotrophic bacteria (19).

The 40-kDa polypeptide was expressed from all clones tested, except pDW11, suggesting that it was encoded downstream of the putative *moxI* but within the second *Eco*RI site shown in Fig. 1. In *P. denitrificans*, a gene designated *moxR* that encodes a nonsecreted polypeptide of 37 kDa (37) is present immediately downstream of *moxI*.

Only one polypeptide (of 24 kDa) was expressed from the clone containing the 10.5-kb *Hin*dIII in the opposite orientation with respect to the T7 promoter (right to left as shown in Fig. 1) that was not present in controls. This polypeptide was not present in any other tested clones and therefore must be encoded within the right *Eco*RI-*Hin*dIII fragment shown in Fig. 1.

Immunoblotting. Antiserum to the *Methylomonas* sp. strain A4 methanol dehydrogenase holoenzyme that cross-reacted to both the large and small subunits (MoxF and MoxI, respectively) of *Methylomonas* sp. strain A4 methanol dehydrogenase was generated (Fig. 4A). This antiserum and antiserum against *Methylomonas* sp. strain A4 cytochrome c_{552} (12) were used in immunoblotting experiments to identify these polypeptides in *E. coli* expression extracts. The low level of expression obtained with the labelling protocol used for the extracts shown in Fig. 5 was not sufficient to detect polypeptides with antiserum. Therefore, separate experiments were carried out to obtain high-level protein expression, using a protocol omitting [^{35}S]methionine and inducing for 2.5 h instead of 20 to 30 min (see Materials and Methods). The level of induction was not sufficient to observe these expressed polypeptides above the background of *E. coli* polypeptides on polyacrylamide gels. However, they were detectable with antiserum (Fig. 4).

The antiserum to methanol dehydrogenase cross-reacts with a number of polypeptides in *E. coli* extracts, including one that is slightly larger than the mature form of the methanol dehydrogenase large subunit (Fig. 4A and B). However, by comparing the lanes showing expression extracts from cells containing clones with the 10.5-kb *Hin*dIII insert in incorrect (pDW8A) and correct (pDW8B) orientations (Fig. 4A and B, lanes 1 and 2, respectively), it is clear that a doublet of polypeptides at approximately 66 and 64.5 kDa is detected. The smaller of these comigrates with purified methanol dehydrogenase large subunit (Fig. 4A and B, lanes 7 and 5, respectively), confirming the identity of these polypeptides as forms of MoxF, presumably the unprocessed and processed forms. Likewise, the expression extract from the cells with a clone containing the 2.2-kb *Eco*RI fragment (pDW11; Fig. 4A and B, lanes 3) shows a dark band corresponding to a polypeptide of approximately 57 kDa, with other bands above and below, confirming that this is a truncated form of MoxF. The upper band probably represents the unprocessed form and the lower bands are probably degradation products, which have been observed for MoxF from *M. extorquens* AM1 (28). As expected, the other extracts did not reveal significant bands that corresponded to MoxF.

Expression extracts from cells containing three clones (pDW8B, pDW33, and pDW33ΔBal) showed cross-reacting bands that comigrated with the methanol dehydrogenase small subunit (MoxI). Purified MoxI from *Methylomonas* sp.

strain A4 appears as a doublet on SDS-polyacrylamide gels (Fig. 4A and B, lanes 8 and 6, respectively). However, in expression extracts from cells containing pDW8B, a triplet of bands (Fig. 4A and B, lanes 2) probably corresponding to the unprocessed form (upper band) and the two processed forms (lower bands) is observed. A similar triplet is observed in expression extracts of cells containing pDW33ΔBal (Fig. 4A, lane 5), although the amount was lower and the upper band is very faint. A very faint band was also observed in expression extracts of cells containing pDW33 (Fig. 4B, lane 4), confirming that MoxI was expressed from this clone. The relatively low level of expression from pDW33 and pDW33ΔBal compared with that observed in Fig. 5 is probably due to the instability of MoxI in the absence of MoxF during the longer induction period. A similar phenomenon was observed for *M. extorquens* AM1 *moxI* expression (3).

The antiserum against *Methylomonas* sp. strain A4 cytochrome c_{552} detected a number of bands in the purified preparation (Fig. 4C, lane 5) which represent multimers of the cytochrome that form during storage (11a). The smallest of the bands detected is the monomer and corresponds to a molecular mass of approximately 14 kDa (13). A band of similar molecular mass was detected in expression extracts from cells containing pDW8B and pDW33, although the band was faint in the latter case. No upper band corresponding to the unprocessed form was observed, but, again, this may be due to differences resulting from the long induction time for these experiments compared with those from Fig. 5.

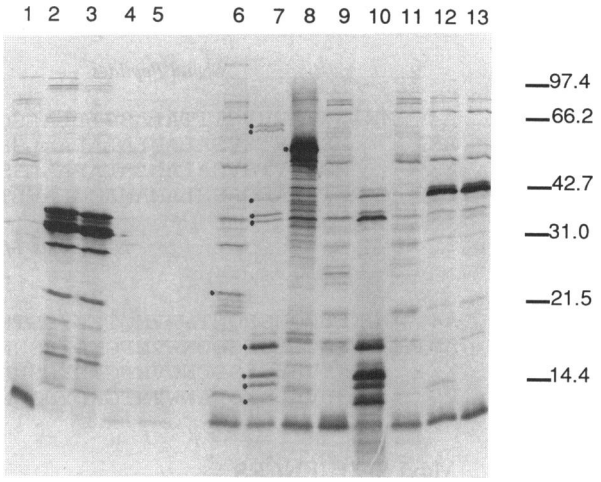

FIG. 5. Autoradiogram of polypeptides expressed by the T7 expression system in *E. coli* strains containing pGP1-2 (plasmid control) (lane 1), pT7-3 (vector control) (lane 2), pT7-4 (T7 RNA polymerase vector control) (lane 3), pT7-5 (vector control) (lane 4), pT7-6 (vector control) (lane 5), pDW8A (10.5-kb *Hin*dIII fragment in pT7-6; orientation with respect to the T7 promoter is right to left, as shown in Fig. 1) (lane 6), pDW8B (10.5-kb *Hin*dIII fragment in pT7-6; orientation with respect to the T7 promoter is left to right, as shown in Fig. 1) (lane 7), pDW11 (2.2-kb *Eco*RI fragment in pT7-6; orientation with respect to the T7 promoter is left to right, as shown in Fig. 1) (lane 8), pDW12 (2.2-kb *Eco*RI fragment in pT7-6; orientation with respect to the T7 promoter is right to left, as shown in Fig. 1) (lane 9), pDW33 (3.5-kb *Eco*RI fragment in pT7-6; orientation with respect to the T7 promoter is left to right, as shown in Fig. 1) (lane 10), pDW13 (3.5-kb *Eco*RI fragment in pT7-6; orientation with respect to the T7 promoter is right to left, as shown in Fig. 1) (lane 11), pDW33ΔBal (deletion of proximal *Bal*I fragment from pDW33) (lane 12), and pDW33ΔSph (deletion of proximal *Sph*I fragment from pDW33) (lane 13). Standards (in kilodaltons) are indicated on the right, and the major expressed polypeptides are marked with dots.

DISCUSSION

Methanol oxidation genes have previously been studied for facultative methanol utilizers and type II methanotrophs, which are all members of the α subgroup of the *Proteobacteria* (8). The data for these bacteria suggest that the structural genes for methanol dehydrogenase (*moxF* and *moxI*) are clustered with the gene for the methanol dehydrogenase-specific cytochrome *c* (*moxG*) and two genes of unknown function (*moxJ* and *moxR*) in the order *moxFJGIR* (19). So far, *moxJ* and *moxR* have been demonstrated to be present only in *M. extorquens* AM1 and *P. denitrificans* (3, 4, 37). We now report the first study of this *mox* gene cluster in the type I marine methanotroph *Methylomonas* sp. strain A4, a member of the γ group of the *Proteobacteria* (8). Our data suggest that in this more distantly related methylotroph, a similar *moxFJGIR* gene cluster is present.

This gene cluster is present on a 10.5-kb DNA fragment cloned from *Methylomonas* sp. strain A4 with a *moxF* hybridization probe from *M. extorquens* AM1. The gene encoding the large subunit of methanol dehydrogenase (*moxF*) has been identified on this DNA fragment by expression and immunoblotting experiments as well as by sequencing of the 5′ region. The translational start site of *moxF* was located approximately 0.7 kb from the left end of the fragment shown in Fig. 1, so *moxF* is expected to extend an additional 1.7 kb to approximately 0.3 kb beyond the second *Eco*RI site shown in Fig. 1.

Expression and immunoblotting experiments suggest that four other genes are present downstream of *moxF*, including, in order, the putative *moxJ*, *moxG*, *moxI*, and the putative *moxR* (Fig. 1). The four polypeptides that define the genes downstream of *moxF* were found to be encoded within a 3.5-kb *Eco*RI fragment. This fragment is just large enough to encode all four polypeptides, suggesting that the genes are closely spaced, as shown in Fig. 1. *moxI* encodes the methanol dehydrogenase small subunit, as confirmed by expression and immunoblotting experiments. *moxG* encodes the methanol dehydrogenase-specific cytochrome. Four soluble cytochromes *c* have been purified from *Methylomonas* sp. strain A4, and it has been suggested that cytochrome c_{552} is MoxG (13). The expression and immunoblotting studies reported here confirm that cytochrome c_{552} is the *moxG* gene product.

The assignments of *moxJ* and *moxR* are tentative and are based on expression experiments that show gene products with molecular masses similar to that of MoxJ from *M. extorquens* AM1 and *P. denitrificans* (3, 4, 37) and that of MoxR from *P. denitrificans* (37). However, the position of these genes and the observation that the putative periplasmic polypeptide MoxJ was expressed as a doublet, while the putative cytoplasmic MoxR was expressed as a single polypeptide, strongly suggest that these are indeed the corresponding homologs. Our data were not sufficient to determine the order for *moxJ* and *moxG*, but, in analogy to *M. extorquens* AM1 and *P. denitrificans*, it seems likely that they are as shown in Fig. 1.

It would be useful to confirm the role of these *mox* genes by generating chromosomal mutations in each of them and characterizing the mutants. In facultative methylotrophs, this has been accomplished by recombinational insertion mutagenesis from cloned genes (28, 37). However, this is not

MoxF SEQUENCES

Signal Peptides

```
A4   MRISKFARHVGTTTALASGILLALSQPAQA -
AM1    MSRFVTSVSALAMLALAPAALSSGAYA -
XX     MSRFVTSVSALAMLALAPAALSSVAYA -
PD   MNRNTPKARGASSLAMAMAMGLAVLTTAPA -
```

N-terminus, Mature Polypeptides

```
           10        20        30        40        50        60
A4   NKELDQMSRQNTNWVMQTKDYSSTHYSELYDINITNVKNLKPAWSFSTGVLNGHEGGPLVVD
AM1  NDKLVELSKSDDNWVMPGKDYDSNNPSDLKQINKGNVKQLRPAWTFSTGLLNGHEGAPLVVD
XX   NDKLVELSKSDDNWVMPGKDYDSNNYSDLKQVNKSNVKQLRPAWTFSTGLLNGHEGAPLVVD
PD   NDQLVELAKDPANWVMTGRDYNAQNTSEMTDINKENVKQLRPAWSFSTGVLHGHEGTPLVVG
```

MoxI SEQUENCES

N-Terminus, Mature Polypeptides

```
          5    10   15
AM1  YDGTKCKAAGNCWEPKPGF
A4   YDGTNCK-PGVCWEPKPGY
PD   YDGTNCKAPGNCWEPKPDY
```

FIG. 6. Comparison of the N-terminal amino acids for MoxF and MoxI from *Methylomonas* sp. strain A4 to the same portions of MoxF and MoxI from facultative methylotrophs. For the mature polypeptides, residues conserved in all four sequences are shown in boldface type. Abbreviations: A4, *Methylomonas* sp. strain A4; AM1, *M. extorquens* AM1 (data from reference 4); XX, *M. organophilum* XX (data from reference 23); PD, *P. denitrificans* (data from reference 16).

possible in *Methylomonas* sp. strain A4 since the organism is an obligate methylotroph and Mox mutants would be lethal. We have not been successful with heterologous complementation experiments with *moxF* and *moxG* mutations of *M. extorquens* AM1 and pDW8B (11b), presumably because of problems of expression, problems of functional incompatibility, or both.

The partial N-terminal sequence of MoxF reported here is the first for a methanotroph. As shown in Fig. 6, substantial identity exists between the N-terminal portions of mature MoxF from facultative methylotrophs and from *Methylomonas* sp. strain A4. Thirty of 62 residues were identical in all four cases. However, the overall conservation in this region is less than that between MoxF from the three facultative methylotrophs, in which 39 residues were identical. Eighteen amino acids of N-terminal sequence from MoxI of *Methylomonas* sp. strain A4 are reported in this article, and these also show high identity to other known MoxI sequences, with 12 of 19 amino acids identical in all three sequences (Fig. 6).

The similarity between the *moxFJGIR* gene clusters of facultative methylotrophs and *Methylomonas* sp. strain A4 noted above does not extend to the promoter region. Although the promoter for *moxF* has not been confirmed for the facultative methylotrophs, the transcriptional start site for *moxF* has been determined for *M. extorquens* AM1 (4) and *Methylobacterium organophilum* XX (23). In both cases, a conserved sequence (AAAGACA–18 bp–TAGAA A–5 bp–+1 [19]) that might represent a promoter is present at the −10 and −35 positions. A similar sequence is present upstream of *moxF* in *P. denitrificans* (37), but the transcriptional start site has not yet been reported. Although a similar sequence is also present upstream of *moxF* from *Methylomonas* sp. strain A4, it begins 45 nucleotides from the transcriptional start site, which is probably too distant for the sequence to be a promoter (Fig. 2). Although a perfect consensus σ^{70} −35 sequence (17, 21) is present at position −32, nothing similar to the consensus −10 sequence is present in the correct location. The sequence directly 5′ to the transcriptional start site bears no resemblance to promoters dependent on σ^{54} or σ^{32} (17) or to consensus *Pseudomonas* promoters (11), so it is not clear what the promoter sequence might be. Further work with gene fusions and site-directed mutagenesis will be necessary to define the *moxF* promoter, but it is clear that it is different from the *moxF* promoter sequence of facultative methylotrophs. This difference may reflect the disparate regulatory strategies of the two groups. In the facultative methylotrophs, the methanol oxidation rate is an order of magnitude greater in cells grown on one-carbon compounds than in cells grown on multicarbon compounds (19). However, the methanotrophs are obligate methylotrophs and do not regulate their methanol oxidation activity in response to the carbon source (19). The cloning and analysis of the *moxFJGIR* region from a type I methanotroph will now allow a detailed comparison of transcription and transcriptional regulation of methanol oxidation in facultative methylotrophs and obligate methanotrophs.

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